Brief Report

Steroid-resistant nephrotic syndrome caused by co-inheritance of mutations at *NPHS1* and *ADCK4* genes in two Chinese siblings

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Summary	Hereditary nephrotic syndrome often presents with steroid-resistance and onset within the first year of life. Mutations in genes highly expressed in podocytes have been found in two thirds of these patients, especially *NPHS1* and *NPHS2* among at least 29 genetic causes that have been discovered. We reported two siblings with steroid-resistant nephrotic syndrome caused by co-inheritance of mutations at *NPHS1* (c.1339G>A, p.E447K) and *ACDK4* (c.748G>C, p.D250H) genes. The siblings presented with steroid-resistant nephrotic syndrome and pathological lesions of focal segmental glomerulosclerosis (FSGS), while the elder sister also developed hypertension, renal failure and cardiac dysfunction.

Keywords: Steroid-resistant nephrotic syndrome, *NPHS1*, *ADCK4*, China

1. Introduction

Nephrotic syndrome is the most common glomerular disease encountered during childhood. It is characterised by significant proteinuria (early morning urine protein with creatinine ratio greater than 200 mg/mmol) leading to hypoalbuminaemia (plasma albumin of less than 25 g/L). Following the reduction in circulating proteins there is a drop in plasma oncotic pressure which manifests as generalised edema. The clinical triad of edema, nephrotic range proteinuria and hypoalbuminaemia defines nephrotic syndrome. This triad is typically accompanied by dyslipidaemia with elevated plasma cholesterol and triglycerides (*1*).

Nephrotic syndrome can be classified by the etiology into primary (hereditary), secondary and idiopathic one. Hereditary nephrotic syndrome often presents with steroid-resistance and onset within the first year of life, sometimes beginning at late childhood or even adulthood. Mutations in genes highly expressed in podocytes have been found in two thirds of these patients, especially *NPHS1*, *NPHS2*, *WT1*, *LAMB2* and *ADCK4* among the discovered at least 29 genetic causes (*2-7*).

We reported here steroid-resistant nephrotic syndrome caused by co-inheritance of mutations at *NPHS1* and *ACDK4* genes in two Chinese siblings.

2. Materials and Methods

2.1. *Participants*

This work was carried out with human research ethics approval from the Peking University First Hospital, and it followed the guidelines of the 2000 Declaration of Helsinki and the Declaration of Istanbul 2008. All patients and their family members gave their consent for inclusion in this study.

2.2. *Methods*

Genetic analysis was performed in the genetics laboratories of MyGenostics biotechnology companies in China, using "the hereditary nephrotic syndrome panel" which includes *NPHS1*, *NPHS2*, *ADCK3*, *ADCK4*, *PLCE1*, *WT1*, *CD2AP*, *ACTN4*, *TRPC6*, *INF2*, *LMX1B*, *LAMB2*, *GLA*, *ITGB4*, *SCARB2*, *COQ2*, *COQ4*, *COQ9*, *PDSS2*, *MTTL1*, *CRB2*, *MIF*, *APOL1*, and *SMARCAL1* (*8,9*). In order to exclude Alport syndrome, *COL4A3*, *COL4A4*, and *COL4A5* gene were also analyzed.

Genomic DNA was isolated from peripheral blood of the siblings and their parents using the salting-out method. Target sequences of exons and their flanking

sequences about 50 bp were captured using Agilent probes and sequenced using a NGS sequencer Genome Analyser IIx (GAIIx, Illumina) and Illumina/Solexa platforms. The expected segregation of putative mutations was confirmed in families, whenever possible, and their absence was confirmed in SNPs databases of common benign variants (*http://exac. broadinstitute.org/*, *http://www.1000genomes.org/*, *http://snp.ims.u-tokyo.ac.jp* and *http://www.ncbi.nlm. nih.gov/projects/SNP/*). Human Splicing Finder (*http:// www.umd.be/HSF/*) and Mutation tasting (*http:// mutationtaster.org/*) were used for analysis on splicing mutation and other mutations, respectively. If a novel mutation was found, 100 normal Chinese controls would be examined directly using PCR amplification and sequencing techniques.

2.3. *Case presentation*

Case 1: An 11-year-old girl (elder sister) was hospitalized to our hospital on October 11, 2013 with the complaint of edema and proteinuria for 22 months, dyspnea and weakness for 8 months. She was found with edema 22 months ago, which wasn't taken seriously. Dyspnea and weakness appeared 8 months ago. Laboratory tests in local hospital showed anemia (hemoglobin 77.3 g/L), abnormal renal function (Scr 927 μmol/L, BUN 23 mol/L), proteinuria (174 mg/kg/24h) and hypoalbuminemia (25 g/L). Renal biopsy yielded 33 glomeruli under light microscopy, which included 4 globally sclerotic glomeruli, the others showed mesengial hypercellularity and matrix hyperplasia with 6 focal segmental sclerosis, tubular epithelia vacuolar and granular degeneration without atrophic tubules and interstitial fibrosis. Immunohistochemical studies showed moderate granular C3 and IgM staining of the basal membranes and mesangium. Electron microscopy revealed glomerular basement membrane (GBM) to be not abnormal; no electron-dense deposits were identified within the mesangial and GBM; mesangial hypercellularity and matrix increase combined with sclerosis were present. She was given hemodialysis and maintenance therapy. Her anuria lasted for three months, and she came to our hospital for further diagnosis and treatment.

There was no abnormal birth and past medical history, but a consanguineous family history of father and mother being first cousins.

At admission, her blood pressure was high (140/90 mmHg). Her height was 140 cm, weight was 30 kg and pulse was 120 beats per minute. Physical examination showed cardiac dilatation with a grade 2 precordial systolic murmur, hepatomegaly and splenomegaly (6 and 5 cm below rib cage, respectively). No neurodevelopmental and ophthalmologic deficits were observed. Laboratory tests in our hospital revealed hemoglobin to be 90 g/L, Scr 739 μmol/L and BUN 22. 2 mol/L; type B natriuretic peptide was 4982 pg/

mL. Her liver function was normal. There was no evidence of virus infection such as Epstein-Barr virus, cytomegalovirus or hepatitis virus. Autoimmune profile was within normal range including compliments, anti-nuclear antibody, anti-double-stranded DNA antibody, and anti-neutrophil cytoplasmic antibodies. Ultra-sonography and MRI showed atrophy of renal bodies (6.0 × 2.5 *vs*. 6.0 × 2.2 cm), the border of cortex and medulla was not clear but without polycystic lesions. No cysts were found in liver and pancreas. Echocardiography showed left ventricular enlargement and hypertrophy, with a decreased ejection fraction (EF) value of 10 percent.

Because their parents were consanguineous and her litter brother (see case 2) also had steroid-resistant nephrotic syndrome, hereditary nephrotic syndrome was suspected.

Case 2: A 2-year and-7-month-old boy (litter brother) was hospitalized to our hospital on November 07, 2013 with a complaint of edema and proteinuria for 7 months. Laboratory tests in the local hospital showed proteinuria (189 mg/kg/24h), hypoalbuminaemia (22.5 g/L) hypercholesterolemia (6.12 mmol/L) and microscopic hematuria (RBC 10~30/HP). His renal function was abnormal (Scr 28 μmol/L and BUN 2.6 mol/L). He was treated for nephrotic syndrome with full dose of prednisone for 8 weeks without response. The subsequent courses of cyclophosphamide pulse therapy (total 150mg/kg) proved futile. He came to our hospital together with his elder sister for further diagnosis and treatment.

There was no abnormal birth and past medical history, but a consanguineous family history of father and mother being first cousins.

At admission, his blood pressure was normal (95/65 mmHg). His height and weight were normal. Physical examination showed no positive signs. No neurodevelopmental and ophthalmologic deficits were observed. Laboratory tests in our hospital revealed heavy proteinuria (158 mg/kg/24h); urine microalbumin was 2290 while α1-microglobin was 18.6 mg/L. Albuminaemia (23.8 g/L) and cholesterolemia (8.45 mmol/L) were also shown positive. Scr was 28 μmol/L, BUN was 3.1 mol/L and hemoglobin was 140 g/L. There was no evidence of virus infection such as Epstein-Barr virus, cytomegalovirus or hepatitis virus. Autoimmune profile was within normal range including compliments, anti-nuclear antibody, anti-double-stranded DNA antibody, and anti-neutrophil cytoplasmic antibodies. Ultra-sonography and MRI showed enlarged renal bodies (7.5 × 3.2 *vs*. 7.3 × 3.0 cm), the border of cortex and medulla was not clear, with enhanced echo and without polycystic lesions. No cysts were found in liver and pancreas. Brain MRI showed no abnormal findings. Echocardiography showed normal heart function with an EF value of 69 percent. Renal biopsy yielded 46 glomeruli under

Table 1. Genetic analysis results of the families

Gene	Case 1	Case 2	Father	Mother
NPHS1	c.1339G>A (p.E447K), Hom*	c.1339G>A (p.E447K) Hom	c.1339G>A (p.E447K) Het*	c.1339G>A (p.E447K) Het
ADCK4	c.748G>C (p.D250H) Hom	c.748G>C (p.D250H) Hom	c.748G>C (p.D250H) Het	c.748G>C (p.D250H) Het

*: Hom: homozygous; Het: heterozygous.

light microscopy, which included 3 globally sclerotic glomeruli, the others showed mesengial hypercellularity and matrix hyperplasia with 5 focal segmental sclerosis, tubular epithelia vacuolar and granular degeneration with interstitial fibrosis. Immunohistochemical studies showed moderate granular C3 (1+ ~ 2+) staining of the mesangium. Electron microscopy revealed irregular thickening and segmental layering of the glomerular basement membrane, segmental fusion of the epithelial foot process; no electron-dense deposits were identified; mesangial hypercellularity and matrix increase combined with sclerosis were present.

Because their parents were consanguineous and his elder sister (see case 1) also had steroid-resistant nephrotic syndrome, hereditary nephrotic syndrome was suspected.

3. Results and Discussion

Genetic analysis results showed that both of the siblings carried a homozygous mutation in exon 11 c.1339G>A (p.E447K) in the *NPHS1* gene and a homozygous mutation in exon 9 c.748G>C (p.D250H) in the *ADCK4* gene. Their father and mother carried the same heterozygous mutations as the siblings. The parents showed no proteinuria, hematuria, and their renal function was normal. No variations were found on *NPHS2*, *WT1*, *COL4A3*, *COL4A4* and other associated genes. See Table 1.

The p.E447K mutation of *NPHS1* was found in the SNPs databases rs28939695, A = 0.0070/35 and 0.002898/347 in 1000Genomes and ExAX, respectively. Mutation tasting analysis showed amino acid sequence changed heterozygously in TGP or ExAC, known disease mutation at this position (HGMD CM004008), protein features might be affected, known disease mutation could be pathogenic. And it was not found in 100 normal Chinese controls.

The p.D250H mutation of ADCK4 was found 3 in ExAX (19-41209497-C-T, A = 2.514e-05) but not in 1000Genomes. Mutation tasting analysis showed that amino acid sequence was changed, protein features might be affected. And also it was not found in 100 normal Chinese controls.

The elder sister was changed from hemodialysis to peritoneal dialysis, given metoprolol tartrate, losartan potassium, coenzyme Q10 synthetic and maintenance therapy. With a follow-up of 24 months, her renal function remained stable (Scr 368 ~ 453 μmol/L, BUN 12.5 ~ 18.8 mol/L) and blood pressure was normal (100 ~ 120/75 ~ 90 mmHg) while her heart function showed no improvement (EF value of 15 ~ 25 percent).

The litter brother was treated with prednisone and tacrolimus for 6 months, his proteinuria varied from 86 to 136 mg/kg/24h. Prednisone and tacrolimus were withdrawn after the genetic diagnosis, he was also given coenzyme Q10 synthetic and maintenance therapy. With a follow up of 24 months, his renal function showed a slight increase (Scr 45 ~ 56 μmol/L, BUN 6.8 ~ 11.5 mol/L), blood pressure remained normal (90 ~ 115/60~85 mmHg), proteinuria varied between 86~136 mg/kg/24h, heart functionwas normal (EF value of 62 ~ 71 percent).

Nephrotic syndrome (NS) is a clinicopathological entity characterized by proteinuria, hypoalbuminemia, peripheral edema, and hyperlipidemia. It is the most common cause of glomerular disease in children and adults. Classically, 80% of cases in the pediatric age group are steroid sensitive nephrotic syndrome (SSNS), while the remaining 20% are called steroid-resistant nephrotic syndrome (SRNS). SRNS is characterized by a rapid progression to end-stage kidney disease (ESKD) with apathological lesion of focal and segmental glomerulosclerosis (FSGS), and it is the most common glomerular cause of ESKD (*10*). Inherited structural defects of the glomerular filtration barrier are responsible for a large proportion of SRNS cases (*2-7*). Classically, mutations in the *NPHS1* and *NPHS2* genes have been distinguished by their implications in familial congenital (onset at birth to 3 month) and in childhood-onset (later than 3 month) cases, respectively (*5,11*). Furthermore, it has recently been shown that mutations in *NPHS1* also account for a nonnegligible proportion of infantile, childhood and adult-onset SRNS cases (*12-14*). *ADCK4* gene, which located on chromosome 19q13.2 and encodes the aarF domain containing kinase 4, is now well-known as a single-gene cause of SRNS (*15-17*).

However, there was no report on SRNS caused by co-inheritance of mutations of *NPHS1* and *ACDK4* genes.

Our two siblings both presented with SRNS and pathological lesions of focal segmental glomerulosclerosis (FSGS), the elder sister also had hypertension, renal failure and cardiac dysfunction. They were from a consanguineous family. Genetic analysis showed that both of the siblings carried a homozygous mutation c.1339G>A (p. E447K) in the *NPHS1* gene and a homozygous mutation c.748G>C (p. D250H) in the *ADCK4* gene. Their father and mother carried the same

mutations heterozygously as the siblings but without any abnormal manifestations.

Both of the mutations are analyzed by Mutation tasting to be disease-causing. The E447K mutation of *NPHS1* had been reported previously in congenital nephrotic syndrome (CNS), which is within motif Ig5 and is unlikely to affect the function of nephrin because both glutamic acid and lysine are hydrophilic (*18*). However, the onset ages of the siblings are 9-year and 2-year old respectively, they are childhood and infantile nephrotic syndrome but not CNS. The onset ages of SRNS caused by *ADCK4* mutation are generally late that range from 7 ~ 21 years old when patients develope ESRD to 7 ~ 23 years old when they were found with a renal pathology of FSGS (*16*). ADCK4 is thought to be an important differential diagnosis to consider in case of adolescent-onset multidrug-resistant proteinuria with FSGS on biopsy (*15*). We are not sure which mutation is the main genetic cause of our SRNS siblings although their pathologic results are both FSGS.

There are reports on digenic inheritance of *NPHS1* and *NPHS2* mutations in congenital FSGS (*19*), *TRPC6* and *NPHS1* mutations in FSGS (*20*), respectively. We confirm an overlap in the *NPHS1/ADCK4* mutation spectrum with a unique characterization which results in a second hit and appears to modify the phenotype. This may result from an epistatic gene interaction, and provides a rare example of multiple allelic hits being able to modify an autosomal recessive disease phenotype in humans. The elder sister showed ESRD and especially severe heart function failure, besides relating to ESKD and hypertension itself, which might be explained by the D250H in *ADCK4* gene. Our findings provide the first evidence for a functional inter-relationship between *NPHS1* and *ADCK4* in human nephrotic disease, thus underscoring their critical role in the regulation of glomerular filtration (*19*).

Coenzyme Q10 (CoQ10) is an essential component of eukaryotic cells and is involved in crucial biochemical reactions such as the production of ATP in the mitochondrial respiratory chain, the biosynthesis of pyrimidines, and the modulation of apoptosis. CoQ10 requires at least 13 genes for its biosynthesis (*21*). Mutations in these genes cause primary CoQ10 deficiency, a clinically and genetically heterogeneous disorder. To date mutations in 8 genes (*PDSS1*, *PDSS2*, *COQ2*, *COQ4*, *COQ6*, *ADCK3*, *ADCK4*, and *COQ9*) have been associated with CoQ10 deficiency presenting with a wide variety of clinical manifestations. Onset can be virtually at any age, although pediatric forms are more common. Symptoms include those typical of respiratory chain disorders (encephalomyopathy, ataxia, lactic acidosis, deafness, retinitis pigmentosa, hypertrophic cardiomyopathy), but some (such as steroid-resistant nephrotic syndrome) are peculiar to this condition (*22*).

Primary CoQ10 deficiency is caused by mutations in *COQ* genes, while secondary deficiencies are related to defects in genes not directly involved in CoQ10 biosynthesis, or to non-genetic factors such as fibromyalgia. The peculiarity of CoQ10 deficiency among mitochondrial disorders is that patients respond well to oral supplementation with CoQ10, making this the only currently treatable mitochondrial disorder. High-dose oral CoQ10 supplementation can stop the progression of the encephalopathy (*23*) and also of the renal manifestations in patients with *COQ2* (*24*), *COQ6* (*25*) and *ADCK4* (*16*) mutations. However, oral CoQ10 supplementation showed no response on heart function in our elder sister, maybe because it was too late for her disease course. It is known that oral CoQ10 supplementation treatment should start as early as possible in the course of the disease, because, although it is possible to stop the progression of the disease, once damage in critical organs such as heart or kidney is established, only minimal recovery is possible (*24*). It needs further follow up to observe whether oral CoQ10 supplementation could delay or prevent ESRD and heart problem in our litter brother. It was a pity that we could not detect the levels of CoQ10 in our two cases.

In conclusion, we were the first to report two childhood SRNS cases with FSGS caused by co-inheritance of mutations at *NPHS1* and *ACDK4* genes in China, one case also developed ESRD and presented with especially severe heart function failure. Our findings provide the first evidence for a phenotype inter-relationship between *NPHS1* and *ADCK4* in human nephrotic disease.

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